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SYMBIOSIS BETWEEN THE ZOOXANTHELLA *SYMBIODINIUM* (= *GYMNODINIUM*) *MICROADRIATICUM* (FREUDENTHAL) AND FOUR SPECIES OF NUDIBRANCHS

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ABSTRACT

The dinoflagellate *Symbiodinium* (= *Gymnodinium*) *microadriaticum* (Freudenthal) occurs in a symbiotic association with the nudibranchs *Melibe pilosa,* an undescribed *Melibe* sp., *Pteraeolidea ianthina,* and *Berghia major.* The algal symbionts reside in host-derived "carrier" cells associated with the host's digestive gland. Longer survival of starved *M. pilosa, P. ianthina,* and *B. major* in constant light than in constant dark indicates that photosynthetically fixed carbon is translocated from symbiont to host. Large lipid deposits, present in the same animal cells that contain zooxanthellae in *Melibe* sp. and *P. ianthina,* suggest that lipid or lipid precursors may comprise part of the translocated nutrients in these species. A large proportion of the fecal material in *Melibe* sp., *P. ianthina,* and *B. major* is composed of degenerate algal cells. It is possible that these species obtain part or all of their translocated nutrients by digestion of some of their algal symbionts. An organ that appears to function as the site of zooxanthella digestion is present in the cerata of *P. ianthina.* Zygotes and larvae of all four nudibranch species are devoid of symbionts, thus each new host generation must be re-infected with *S. microadriaticum.* Adults of *B. major* feed on prey that contain symbiotic zooxanthellae while the other three nudibranch species examined do not. These facts suggest that while the historic inception of the symbiosis in *B. major* was probably secondary, the symbionts being derived from the prey; in *M. pilosa, Melibe* sp., and *P. ianthina* the inception may have been primary, the symbionts being obtained by inadvertant ingestion by the host.

INTRODUCTION

The symbiotic relationships between zooxanthellae and a number of different invertebrate hosts have been of biological interest since the late 1800's (*e.g.,* Brandt, 1881), initially as a zoological curiosity and more recently as a means of studying the interactions between genomes of different evolutionary origins that are in intimate cytoplasmic contact. While a considerable amount of work has been done on tridacnid clams (Kawaguti, 1966; Muscatine, 1967; Fankboner, 1971; Goreau *et al.,* 1973; Fitt and Trench, 1981; Trench *et al.,* 1981) and coelenterates (Boschma, 1925; Muscatine and Hand, 1958; Muscatine, 1967; Taylor, 1969a, b; Trench, 1971a, b, c; 1974; and many others), little consideration has been given to associations between zooxanthellae and various nudibranch species. Inquiries that have investigated this symbiosis have been descriptive in nature and, aside from the recent work of Rudman (1979, 1981a, b, 1982), have lacked detail and comparative analysis (Hornell, 1909; Naville, 1926; Rousseau, 1931). Research concerned with the physiology of nudibranch-zooxanthellae associations is totally lacking in the literature.

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The fact that different species of these complex organisms have varying degrees of morphological modification to accomodate the symbiont (Rudman, 1981a, b; 1982) suggests that host-symbiont interaction is not the same in all nudibranch species. Analysis of the physiological aspects of these associations should allow further insight into the evolution, inception, maintenance, and control of algal-invertebrate symbioses.

At least seven species of nudibranchs found in Hawaiian waters maintain a symbiosis with zooxanthellae. These are the dendronotids *Melibe pilosa,* and two undescribed *Melibe* species, and the aeolids *Berghia major* (possibly the same as *Spirulla major* as re-described by Rudman, 1981b), *Pteraeolidea ianthina, Baeolidea nodosa,* and one unidentified aeolid species. The following research considers the nature of the symbiotic association in *M. pilosa,* one of the undescribed *Melibe* species (hereafter termed *Melibe* sp.), *B. major,* and *P. ianthina.*

Materials and Methods

Collection and maintenance of animals and egg masses

Animals were collected in the field by skin and SCUBA diving and were held in the lab under constant light in beakers or glass finger bowls containing pre-filtered (Millipore Cat. no. AP2504700) sea water (hereafter termed PFSW). Water in these containers was changed daily in most instances. Taxonomic identifications of the described species are based on descriptions given by Kay (1979). Egg masses laid in the lab were incubated through hatching in beakers of aerated PFSW that were changed daily.

Identification of host prey

Prey of the nudibranchs examined in this study were determined from examinations of the fecal material of recently collected animals and/or observations in the lab of feeding on prey items found in each species' habitat. In *Pteraeolidea ianthina,* the nematocysts stored in the cnidosacs of the cerata were also examined and compared to those of various coelenterates found in its habitat.

Algal taxonomy

Taxonomic identification of the zooxanthella associated with the nudibranchs was made by comparison of the alga's morphological characteristics, as seen with the light and electron microscopes, with the descriptions of Freudenthal (1962) and Kevin *et al.* (1969). Zooxanthellae were separated from host tissues by homogenizing specimens in a glass tissue grinder with plastic plunger and then washing by centrifugation as described by Muscatine (1967). The algae were then placed in either sea water or Provasoli's E.S. medium (Provasoli, 1968) in constant dark overnight. Motile stages were examind with a compound microscope the next day after the separated algal cells had been exposed to a few hours of light. Motile stages were also produced by a similar incubation of either host fecal pellets or rotting host tissues in sea water.

Translocation of nutrients from symbiont to host

Starvation experiments were performed in constant light or constant dark to test the hypothesis that nutrients, in the form of photosynthetically fixed carbon, are translocated from the zooxanthellae to the host. Similar-sized pairs of animals were starved in containers filled with 0.45 μm Millipore filtered sea water (hereafter termed MFSW) that contained the antibiotics streptomycin sulfate and penicillin G at con-

centrations of 50 and 60 μg/ml respectively (Switzer-Dunlap and Hadfield, 1977) with (*Melibe pilosa* and *Berghia major*) or without (*Pteraeolidea ianthina*) aeration. Animal containers and medium were changed daily. The wet mass of the starved animals was determined on a regular basis; however, loss of cerata in *M. pilosa* obscured changes in mass for this animal.

Host defecation of healthy and degenerate zooxanthellae

Squashes of fecal material from *Melibe pilosa, Melibe* sp., *Berghia major,* and *Pteraeolidea ianthina* were prepared and the healthy and degenerate algal cells in 10 equal areas from each squash were counted under a compound microscope. Results were converted to percentages of the total number of cells in each area. Cells were considered healthy if they were spherical with a smooth border and contained an identifiable chloroplast and other organelles. Degenerate cells were those that had an irregular cell boarder, were shrunken in appearance, and lacked distinct organellar organization. The significance of the change in the results for fecal counts of *Melibe* sp. that were either a) unstarved or starved for 1 day or b) starved for 3 days was determined by comparing the differences between mean percent healthy and mean percent degenerate algal cells in each animal of the two ranked groups with a Mann-Whitney U test (Seigel, 1956).

Identification of lipid in host tissues

Larvae and small, whole cerata of *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* were relaxed in a mixture of three parts PFSW and one part MFSW saturated with chlorobutanol and then fixed in 10% formalin in sea water. Fixed material was dehydrated through an ethylene glycol:distilled water series (1:3, 1:1, 3:1, 100% ethylene glycol; 5 min/treatment) and then transferred to a saturated solution of Sudan IV in ethylene glycol (Chiffelle and Putt, 1951) for 3 to 8 h (modification of method of Williams-Arnold, 1974). Stained material was rinsed in 100% ethylene glycol to remove excess stain and then examined with a compound microscope.

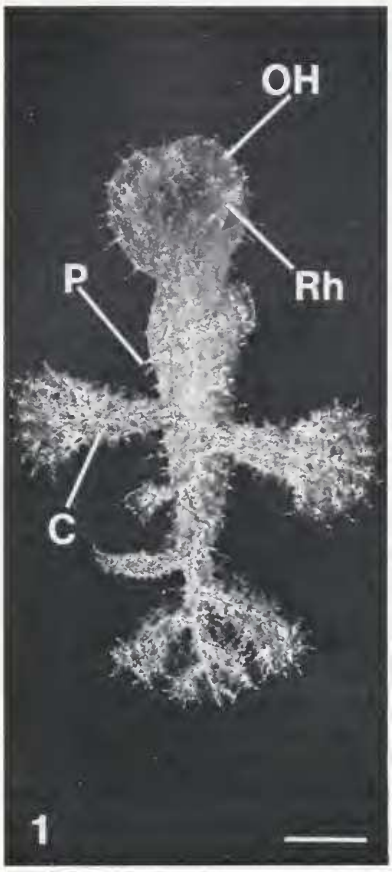
Light and electron microscopy

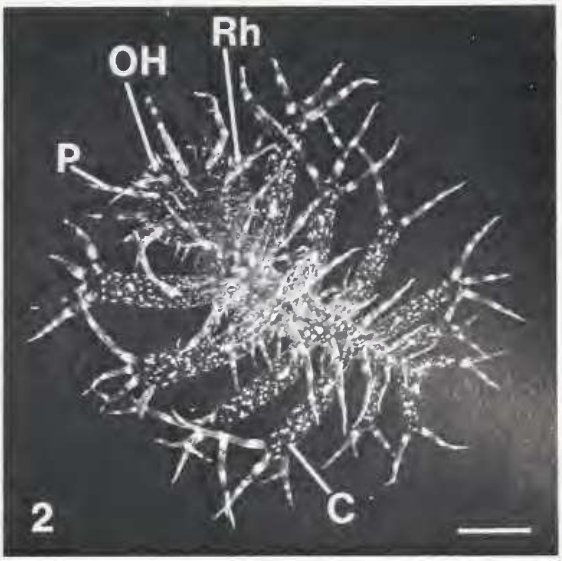
Tissues were relaxed in the chlorobutanol:sea water solution described above and then fixed in 2.5% Millonig's phosphate buffered glutaraldehyde (Cloney and Florey, 1968) for one h. The tissues were then washed in either 2.5% $NaHCO_3$ or a 1:1 mixture of 0.6 *M* NaCl and Millonig's phosphate buffer and post-fixed in 2% OsO_4 in 1.25% $NaHCO_3$ for one h (Wood and Luft, 1965). Fecal pellets of *Melibe* sp. were fixed using the same procedure, but were not placed in the chlorobutanol:sea water mixture. Dehydration was carried out in an ethanol series through 100% propylene oxide and the tissues were embedded in EPON (Luft, 1961). Sections for light microscopy were stained with Richardson's stain (Richardson *et al.,* 1960). Ultrathin sections were stained with uranyl acetate and/or lead citrate.

Results

Host prey

Adults of both *M. pilosa* (Fig. 1) and *Melibe* sp. (Fig. 2) feed on small crustaceans (*i.e.,* copepods, isopods, etc.) that are captured with their expandable oral hood. The exoskeletal remains of these prey are quite evident in the fecal material of freshly collected specimens of both *Melibe* species and their feeding on newly hatched brine





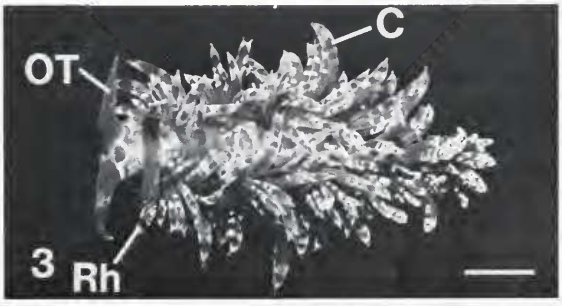


FIGURE 1. Adult *Melibe pilosa* (at least 3 cerata are missing). C–ceras, OH–oral hood, P–papilla, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 2. Unidentified, adult, *Melibe* species. C–ceras, OH–oral hood, P–papilla, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 3. Adult *Berghia major*. C–ceras, OT–oral tentacle, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 4. Adult *Pteraeolidea ianthina*. CT–ceratal tuft, OT–oral tentacle, Rh–rhinophore. Bar ~ 1.0 cm.

shrimp has been observed in the laboratory. *Berghia major* (Fig. 3) collected for this study fed on the swimming anemone *Boloceroides mcmurrichi* that contains zooxanthellae and is common in beds of *Acanthophora spicifera. Pteraeolidea ianthina* (Fig. 4) feeds on various species of hydroids. Observations of *P. ianthina* feeding in the laboratory, of its fecal remains, and of the nematocysts contained in its cnidosacs indicate that at least some of the prey species in the field are *Halocordyle distica* (= *Pennaria tiarella,* Cooke, 1977), a species of *Zanclea* (see Hastings, 1930) and unidentified species from the family Plumulariidae (hydroid taxonomic identifications by W. J. Cooke, University of Hawaii). None of these hydroids (nor others from *P. ianthina*'s habitat that were examined during an extensive survey by the author) contain zooxanthellae. No zooxanthella-containing hydroids, such as *Myrionema ambionenses* (Trench, 1979), are known to occur in Hawaiian waters (W. J. Cooke, pers. comm.). It should be noted that even though *P. ianthina* is often found in apparent association with the zooxanthellae-containing octocoral *Anthelia* (= *Sarcothelia*) *edmondsoni* (Kay, 1979), starved specimens of the aeolid would not feed on this coelenterate when placed with it in aquaria or fingerbowls. Starved *P. ianthina* would feed on the hydroid *Halocordyle distica* under similar circumstances.

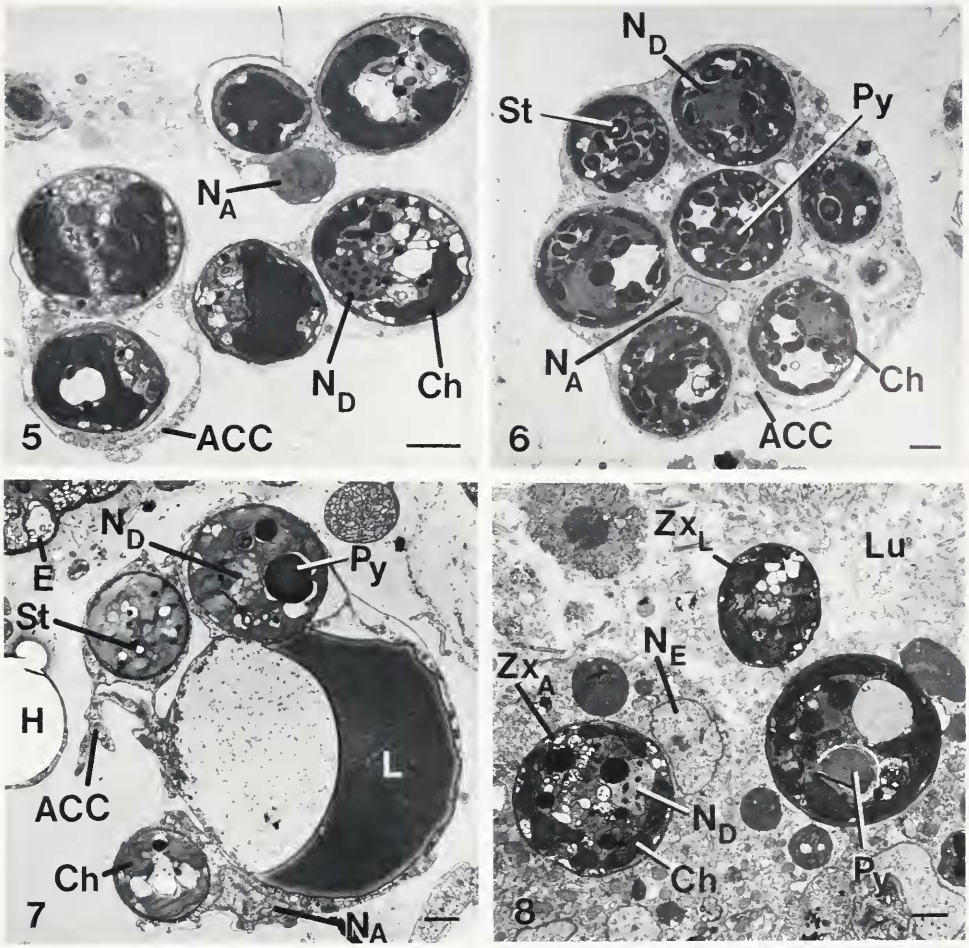
Location of algal symbionts

The egg masses, zygotes, and newly hatched larvae of the four species examined in this study do not contain zooxanthellae. Most of the zooxanthellae found in the adults of all four nudibranch species are intracellular and appear to reside within vacuoles in the cytoplasm of animal "carrier" cells (Figs. 5–8). Those algae that are not intracellular are found in the lumina of the digestive organs. In *P. ianthina* most of the "carrier" cells reside in a presumably protein matrix that forms a layer directly beneath the epidermis in all parts of the body (Fig. 7; also see 16, 17). It should be noted that this layer is not part of the hemocoel. In *Melibe pilosa* and *Melibe* sp. the algal cells, in their respective "carrier" cells, are distributed in a sparsely cellulated matrix that fills each ceras and the rest of the body in areas where specific organs or hemocoel are not located.

The animal cells containing zooxanthellae in all three species appear to have some association with the digestive gland. This association is particularly evident as it applies to extensions of this organ into the cerata. Intracellular zooxanthellae of *Berghia major* occur within epithelial cells of branches of the digestive gland in the cerata (Fig. 8). Algal cells are also found in an apparent proliferation of digestive gland cells that extends into the head region, oral veil, and rhinophores in larger specimens of this species. In all four nudibranch species, more than one algal cell may reside in a given "carrier" cell. Intracellular algal cell division stages have been observed in both species of *Melibe* and in *P. ianthina.*

Taxonomic identification of the algal symbiont

In all four nudibranchs, the vegetive intracellular stage of the alga is identical in morphology to *Symbiodinium* (= *Gymnodinium*) *microadriaticum* Freudenthal (Freudenthal, 1962; Kevin *et al.,* 1969; Taylor, 1974) (Figs. 5, 6, 7, 8). Algal cells artificialy separated from the animal tissue and kept in sea water or Provasoli's E.S. medium overnight form gymnodinoid zoospores like those described by Freudenthal (1962). Motile stages will also arise from zooxanthellae in host fecal pellets or decaying host tissues incubated under similar conditions in sea water.



FIGURES 5–8. Intracellular zooxanthellae in nudibranch ceratal tissues. ACC–animal carrier cell, Ch–chloroplast, N_A–animal carrier cell nucleus, N_D–dinoflagellate cell nucleus, St–starch granule, Py–pyrenoid.

FIGURE 5. *Melibe pilosa.* Bar = 2.0 μm.

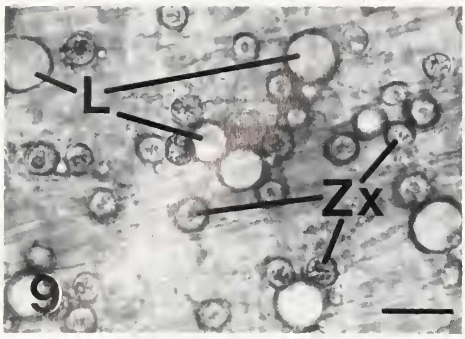
FIGURE 6. Unidentified *Melibe* sp. Bar = 2.0 μm.

FIGURE 7. *Pteraeolidea ianthina.* Algal cells and lipid droplet are within an animal carrier cell that resides in an acellular, presumably protein matrix below the epidermis. Note pyrenoid continuous with chloroplast. E–epidermis, H–hole caused by algal cell knocked out of plastic during sectioning, L–lipid droplet (partially extracted). Bar = 2.0 μm.

FIGURE 8. Intracellular zooxanthellae in presumed digestive cells lining ceratal extensions of the digestive gland in *Berghia major.* Lu–lumen of digestive gland extension into ceras, N_E–nucleus of animal cell in epithelial lining of central extension of digestive gland in ceras, Zx_A–zooxanthella in animal cell of epithilial lining of central extension of digestive gland in ceras, Zx_L–zooxanthella in lumen of central extension of digestive gland in ceras. Bar = 2.0 μm.

Lipid distribution in host tissues

Dense aggregations of refractile droplets are found associated with the intracellular zooxanthellae in adult *Pteraeolidea ianthina* (Fig. 9, also see 7). These droplets stain bright red in whole mounts stained with Sudan IV and green in Richardson's stained plastic sections. A few small droplets that stain red with Sudan IV are also found



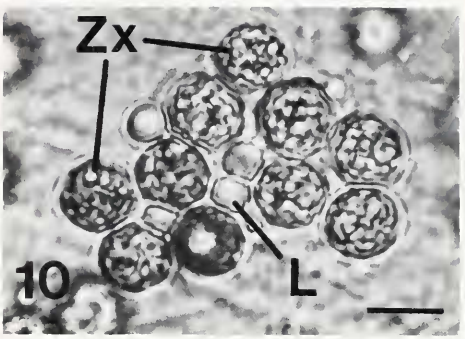


FIGURE 9. Unstained, refractile, lipid droplets in a ceras of *Pteraeolidea ianthina*. L–lipid droplet, Zx–zooxanthellae. Bar = 20 μm.

FIGURE 10. Unstained refractile lipid droplets associated with intracellular zooxanthellae in tissues of *Melibe* sp. L–lipid droplet, Zx–zooxanthellae. Bar = 10 μm.

associated with the intracellular zooxanthellae found in the tissues of adult *Melibe* sp. (Fig. 10). Adult *Melibe pilosa* and *Berghia major* lack refractile droplets.

The newly hatched veligers of all four species contain refractile droplets in their digestive diverticula. The presence of these droplets is particularly evident in newly hatched larvae of *Pteraeolidea ianthina* that contain refractile droplets throughout most larval tissues rather than just in the digestive diverticula. These refractile droplets also stain bright red with Sudan IV in larvae of *Melibe pilosa, Melibe* sp., and *P. ianthina* and green in sections of plastic-embedded larvae stained with Richardson's stain. Larvae of *Berghia major* were not stained.

Translocation of fixed carbon from symbiont to host

Starved adult specimens of *Melibe pilosa, Berghia major,* and *Pteraeolidea ianthina* survive longer in constant light than in constant dark (Table I). Comparative starvation tests were not performed with *Melibe* sp. Starved *M. pilosa* can survive a maximum of 29 days in constant light and 20 days in constant dark. Similar results were obtained for *B. major. Pteraeolidea ianthina,* on the other hand, survive as long as 192 days in constant light and up to 90 days in constant dark. All species tested decrease in mass during starvation. The mode and effects of this mass loss are most evident in *P. ianthina* due to its longer period of survival. Initially a slight increase in mass occurs, followed by a continuous decrease until death. Animals held in constant dark decrease in mass more rapidy than those held in constant light. After 19 days, *P. ianthina* that were initially the same size are still similar in length and appearance regardless of whether they have been starved in constant light or dark. When the same animals are examined after 61 days of starvation, those held in constant dark are considerably smaller than those held in constant light and in an obvious state of mortal decline. The presence of refractile droplets was followed in the cerata of one pair of starved *P. ianthina.* The specimen held in constant light lost all of its refractile droplets after 45 days of starvation and survived a total of 159 days. The animal starved in constant dark lost all of its droplets after 38 days of starvation and survived 74 days.

Host defecation of healthy and degenerate zooxanthellae

In all four species of nudibranchs examined, fecal pellets continue to be extruded throughout periods of starvation. Smears of *Melibe pilosa*'s fecal pellets examined

TABLE I

Effect of starvation in constant light or constant dark on survival in three species of nudibranchs symbiotic with Symbiodinium microadriaticum

Species	Duration of survival (days)	
	Constant light	Constant dark
Berghia major	37	22
Melibe pilosa[a]	25	19
	29	20
	29	20
	$\bar{X} = 28 \pm 2$	$\bar{X} = 20 \pm 1$
Pteraeolidea ianthina[a]	35[b]	74
	147	80
	159	90
	163[c]	81[c]
	192	82
	$\bar{X} = 165 \pm 19$	$\bar{X} = 81 \pm 6$

[a] Survival in constant light is significantly longer than in constant dark. *M. pilosa*, $P = 0.050$; *P. ianthina*, $P = 0.008$; Mann-Whitney U Test (Siegel, 1956).

[b] This animal did not appear to be healthy and was not included in calculation of mean survival time or significance.

[c] These animals were in the process of regenerating their body posterior to the fifth pair of ceratal tufts.

after one day of starvation contain nearly 100% healthy zooanthellae (Table II). Subsequent qualitative examination indicates that *M. pilosa*'s feces continue to be composed of nearly 100% healthy algal cells after several days of starvation. Fecal pellets of starved *Melibe* sp., *Berghia major*, and *Pteraeolidea ianthina* (*e.g.*, Fig. 11) contain large numbers of what are presumably degenerate algal cells. Ultrastructural examination of fecal pellets from starved specimens of *Melibe* sp. reveals both apparently healthy algal cells and algal cells at various stages of degeneration (Figs. 12–14). Counts of healthy and degenerate algal cells with the light microscope show that while about 97% of the algal cells in the feces of starved *M. pilosa* are healthy, over 96% of the algal cells from the feces of starved *Melibe* sp., *Berghia major*, and *Pteraeolidea ianthina* are degenerate (Table II). It is interesting to note that in fecal material from freshly collected (unstarved) *Melibe* sp. about 50% of the algal cells are healthy while after three days of starvation fecal pellets contain nearly 100% degenerate algal cells (Table II). This difference is significant at the $P = 0.001$ level.

The cerata of *Pteraeolidea ianthina* contain dark brown organs (Fig. 15) that are associated with the branches of the digestive gland that extend into the cerata and that contain both healthy and degenerate zooxanthellae. These are the only tissues in which degenerate algal cells were observed in *P. ianthina*. In sectioned material, these organs are continuous with both the sub-epidermal matrix that contains the "carrier" cells and their associated zooxanthellae and refractile droplets, and also with the extension of the digestive gland in each ceras (Figs. 16, 17). Algal cells can be seen within the organ's connection with the sub-epidermal matrix (Fig. 17). Zooxanthellae can sometimes be found within the lumen of the dark brown organ that is continuous with the lumen of the central digestive gland extension in each ceras (Fig. 16). Electron micrographs of sections of the organ show that intracellular zooxanthellae within it are either healthy or at different stages of degeneration. Dense-cored vesicles,

TABLE II

Appearance of zooxanthellae in fecal pellets from nudibranchs symbiotic with zooxanthellae

Species	n[a]	n[b]	# of days of starvation[c]	Zooxanthellae	
				% healthy	% degenerate
Melibe pilosa	1	9	0	$\bar{X} = 78 \pm 29$	$\bar{X} = 22 \pm 29$
	5	10	1	$\bar{X} = 97 \pm 4$	$\bar{X} = 3 \pm 4$
Melibe sp., Recently collected	6	10	0–1	$\bar{X} = 47 \pm 14$	$\bar{X} = 53 \pm 14$
After 3 days of starvation	6	10	3	$\bar{X} = 3 \pm 4$	$\bar{X} = 97 \pm 4$
Pteraeolidea ianthina	2	10	0	$\bar{X} = 2 \pm 4$	$\bar{X} = 98 \pm 4$
	1[d]	10	0	$\bar{X} = 40 \pm 39$	$\bar{X} = 60 \pm 39$
	7	10 or 20	1	$\bar{X} = 2 \pm 1$	$\bar{X} = 98 \pm 1$
	2	10	15	$\bar{X} = 3 \pm 6$	$\bar{X} = 97 \pm 6$
	2	10	22	$\bar{X} = 3 \pm 7$	$\bar{X} = 97 \pm 7$
Berghia major	1	11	?	$\bar{X} = 2 \pm 2$	$\bar{X} = 98 \pm 2$

[a] Number of fecal composition determinations. Each one representing a different animal.

[b] The number of equal sized areas in which the healthy and degenerate zooxanthellae for each fecal determination were counted.

[c] This is the same as the number of days since the animals were field collected.

[d] The feces of this animal contained many hydroid perisarcal remnants suggesting that it had eaten recently and was not starved.

possibly lysosomes (Cook, 1980), are found in the animal cytoplasm surrounding the algal cells (Fig. 18).

DISCUSSION

Location of algal symbionts in host tissues

As reported for numerous symbiotic coelenterates (Boschma, 1925; Muscatine, 1961; 1974; Mclaughlin and Zahl, 1966; Trench 1971a, b, c; Taylor, 1973; and others), the algal symbionts of *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* are contained within animal "carrier" cells that are apparently specialized for this purpose. A similar situation may exist to some extent in *Berghia major* in the anterior proliferation of symbiont containing cells found in larger specimens of this species; however, many of the zooxanthellae present in *B. major* are phagocytosed by and reside in cells of the digestive epithelium lining the ceratal extensions of the digestive gland. Similar to the conclusions of Rudman (1981a; 1982), for various symbiotic aeolid and arminacean nudibranchs, the animal "carrier" cells of the species examined in this study are associated with cells of the digestive gland and presumably are derived from cells of this linage. This conclusion is not unexpected in that the probable mode of infection, both at the historic inception of the symbiosis and at each new generation, is by phagocytotic uptake of ingested algal cells.

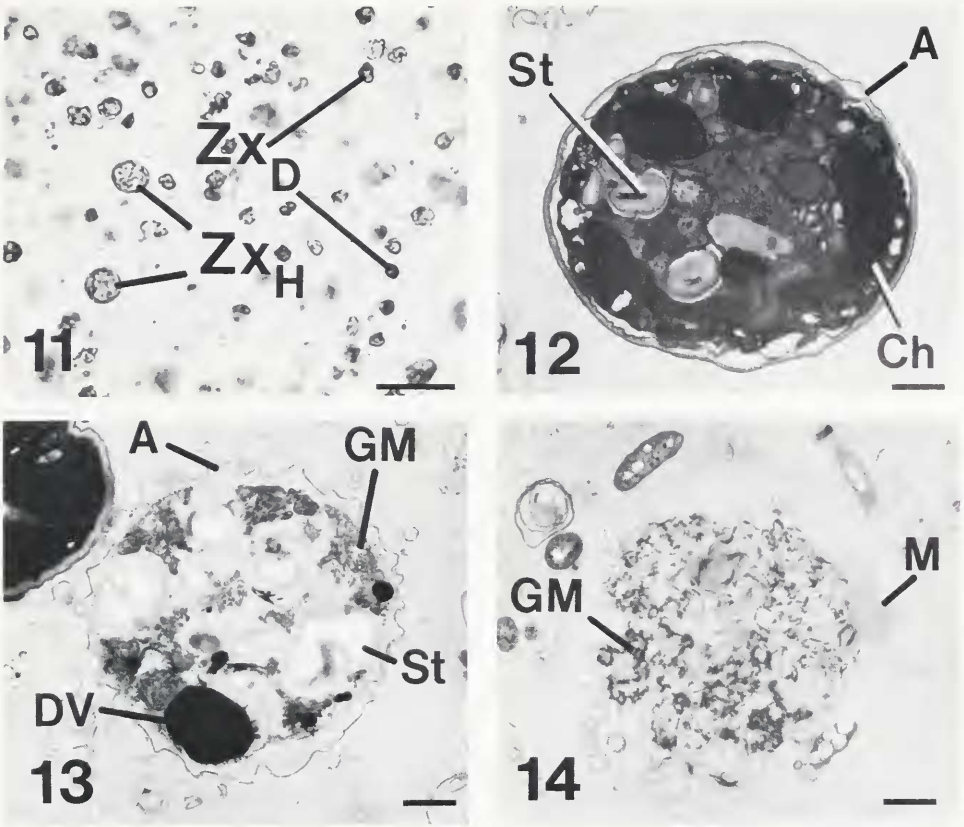


FIGURE 11. Light micrograph of squash of fecal pellet of *Pteraeolidea ianthina* showing presumably degenerate and healthy zooxanthellae. Zx_D–degenerate zooxanthellae, Zx_H–healthy zooxanthellae. Bar = 20 μm.

FIGURES 12–14. Healthy and degenerate zooxanthellae in fecal pellet of *Melibe* sp.

FIGURE 12. Morphologically intact, presumably healthy zooxanthella. Amphiesma is intact and regular, organellar structure is organized, and organelles and components are well defined. A–amphiesma, Ch–chloroplast, St–starch granule. Bar = 1.0 μm.

FIGURE 13. Degenerating zooxanthella. Amphiesma is highly irregular and beginning to break-up. A–amphiesma, DV–dense osmophilic vesicle, GM–granular matrix, St–starch granule. Bar = 1.0 μm.

FIGURE 14. Probable membranous and granular remains of completely degenerated (digested?) zooxanthella. Granular matrix is similar to that within algal cell in Figure 13. Note myelin figures like those described by Trench (1974, p. 204) in degrading zooxanthella of *Zoanthus sociatus*. GM–granular matrix, M–myelin figure. Bar = 1.0 μm.

Translocation of nutrients from symbiont to host

The term "translocation" has been used in previous work to describe the leakage of fixed carbon from algal symbionts to the host tissues (Muscatine and Hand, 1958; Muscatine, 1967; Taylor, 1969a; Trench, 1971a, b, c; Goreau *et al.*, 1973; and others). The implication is that the mechanism for this leakage is a host-mediated transport or diffusion of fixed carbon across the amphiesma of healthy symbionts (Muscatine, 1967; Trench, 1971a, b, c). The results of starvation experiments carried out in the present study lead one to conclude that a similar mechanism for translocation functions in *Melibe pilosa*. Upon initial inspection of starvation results, a similar conclusion

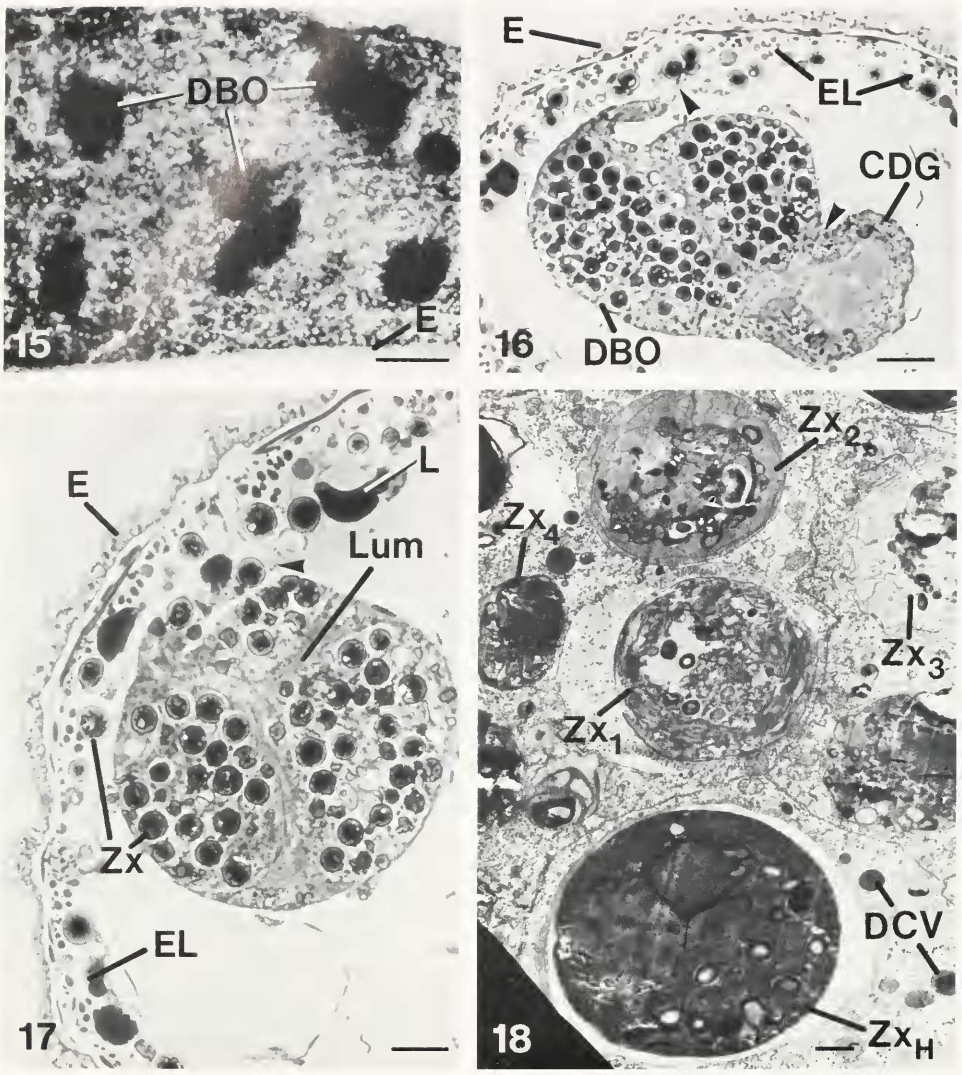


FIGURE 15. The dark brown organs that contain healthy and degenerate zooxanthellae as seen in a light micrograph of part of a whole ceras from *Pteraeolidea ianthina*. DBO–dark brown organs, E–epidermis of ceras, Bar = 200 μm.

FIGURE 16. Phase contrast micrograph of a transverse, Richardson's stained, plastic section of a ceras of *Pteraeolidea ianthina*. The connection of "dark brown organ" to a central extension of the digestive gland in the ceras and to the sub-epidermal layer containing the animal carrier cells and their associated zooxanthellae and lipid droplets can be seen. The lumen within the dark brown organ (see Figure 17) does not extend into the connection with the sub-epidermal layer. CDG–central extension of digestive gland into the ceras, DBO–"dark brown organ," E–epidermis of ceras, EL–sub-epidermal layer containing zooxanthellae and associated lipid droplets within animal carrier cells, Arrowheads–connections of "dark brown organ" with sub-epidermal layer and central extension of digestive diverticulum within ceras. Bar = 30 μm.

FIGURE 17. Phase contast micrograph of a transverse, Richardson's stained, plastic section of a ceras of *Pteraeolidea ianthina* showing zooxanthellae within the sub-epidermal layer's connection with the "dark brown organ." E–epidermis of ceras, L–lipid droplet, Lum–lumen within "dark brown organ." EL–sub-epidermal layer, Zx–zooxanthellae, Arrowhead–connection of "dark brown organ" with sub-epidermal layer (note zooxanthellae within this connective process). Bar = 20 μm.

might be reached for the other nudibranch species examined; however, since large numbers of degenerate algae are present in the feces of *Melibe* sp., *Pteraeolidea ianthina,* and *Berghia major,* it is possible that part or all of the fixed carbon assimilated by these hosts is obtained from the digestion (by host enzymes and/or autolysis) of algal cells (see below). The proportion of fixed carbon attributable to translocation via leakage and/or algal digestion remains to be determined.

Keeble's (1910) suggestion of translocation of "fat" from algal symbiont to host in the acoel flatworm *Convoluta roscoffensis* has recently been confirmed by Meyer *et al.* (1979). Investigations by Patton *et al.* (1977) and Blanquet *et al.* (1979) have demonstrated light dependent translocation of lipid or lipid precursors from symbiotic zooxanthellae to coelenterate hosts. Staining characteristics of the refractile droplets found associated with *Symbiodinium microadriaticum* in the algal "carrier" cells of adult *Melibe* sp. and *Pteraeolidea ianthina* identify the material composing the droplets as lipid. This suggests that translocation of lipid or lipid precursors occurs in these nudibranchs.

Lipid droplets reach very high densities in the tissues of *P. ianthina* and must comprise a substantial energy reserve. Similar high concentrations of lipid in the larvae of this nudibranch [higher concentrations than are found in other opisthobranch larvae (Kempf, 1982)] suggest that some of this lipid is utilized in reproduction.

Digestion of Symbiodinium microadriaticum *by nudibranch hosts*

Digestion of *Symbiodinium microadriaticum* by the invertebrate host has been suggested for tridacnid clams (Yonge, 1936; Fankboner, 1971) and the coelenterates *Astrangia danae* (Boschma, 1925) and *Phyllactis flosculifera* (Steele and Goreau, 1977). Trench (1980) believes that there is not sufficient evidence to support hypotheses of the occurrence of ". . . *in situ* intracellular or extracellular digestion of symbiotic dinoflagellates" in these species. He suggests that previous reports of zooxanthella digestion by hosts may have been due to observations of senescent algal cells that had undergone autolysis (Trench, 1974, 1980). Recently Trench *et al.* (1981) have offered further evidence in support of the autolytic degradation hypothesis for tridacnid species.

The following observations of degenerate algal cells in *Berghia major, Melibe* sp., and *Pteraeolidea ianthina* suggest that these three nudibranchs take an active part in the breakdown of at least some of their algal symbionts: a) the degenerate algal cells that are observed in the feces of these species, b) the near-absence of pycnotic algal cells in the feces of *M. pilosa* while the feces of the other three species examined contain a high percentage of degenerate algal cells, c) the increase in the proportion of degenerate algal cells during starvation of *Melibe* sp. [This is the opposite of what has been observed in *Tridacna gigas* (Trench *et al.,* 1981)], d) the presence of an organ in *P. ianthina* the contents and anatomical position of which indicate its possible function as an algal digestive organ (see below), and e) the extended survival that continues far beyond the point at which algal-associated lipid reserves are depleted when *P. ianthina* is starved in constant light or constant dark. While the research

FIGURE 18. Electron micrograph of a morphologically intact and presumably healthy zooxanthella and different stages of zooxanthella degradation (presumably digestion) within one of the "dark brown organs" found in the cerata of *Pteraeolidea ianthina.* Dense core vesicles, possibly lysosomes, can be seen in the cytoplasm of cells containing zooxanthellae. DCV–dense core vesicle, Zx_H–presumably healthy zooxanthella, Zx_1, Zx_2, Zx_3, Zx_4–stages in the degradation (digestion?) of zooxanthellae in a "dark brown organ" (Zx_3–note apparent breakup of algal wall and plasmalemma). Bar = 1.0 μm.

reported in this study does not conclusively demonstrate that these species enzymatically digest *S. microadriaticum,* this would appear to be one of only two possibilities, the other being that these hosts in some way affect and regulate the autolytic degradation of algal cells.

It is suggested that *Melibe* sp. *Pteraeolidea ianthina* digest some of their algal symbionts, consequently regulating algal density in their tissues (see Taylor, 1969b; Muscatine and Pool, 1979) and at the same time obtaining (via digestive translocation) symbiont-derived nutrients over and above those provided by leakage translocation of fixed carbon and lipid or lipid precursors from healthy algal cells. The increase in the proportion of degenerate algal cells in the feces of *Melibe* sp. shortly after the beginning of starvation suggests that at least this nudibranch can either regulate the rate of algal digestion and perhaps the mitotic rate of its symbionts to its nutritional advantage or that it can control the rate of expulsion of healthy algal cells in its feces. In adult *P. ianthina,* the algal cells, and perhaps lipid droplets, would appear to be removed from their residence in the subepidermal layer and transferred through its connection with the "dark brown organ" to digestive cells within that organ. The indigestible remains of algal cells and a few healthy symbionts would then be exocytosed into the lumen of the organ and subsequently passed out of the animal in its feces. It should be noted that while it is possible that *Berghia major* can digest established symbionts residing in its tissues, it seems more likely that this nudibranch digests mostly excess algal cells derived from its prey as has been reported for *Cuthona poritophages* by Rudman (1981b).

Infection of nudibranch hosts with Symbiodinium microadriaticum

Since the newly hatched larvae of all the nudibranchs examined in this study lack algal symbionts, one must conclude that the symbiosis is re-established in each new generation of host organisms. In *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina,* nudibranchs that do not feed on prey symbiotic with zooxanthellae as adults, it seems probable that this reinfection occurs in one or more of the following ways: 1) by inadvertant ingestion of *Symbiodinium microadriaticum* during larval or post-metamorphic feeding (LaBarbara, 1975; Fitt and Trench, 1981), 2) by juvenile feeding on a food source that contains the symbionts and is different than the prey of the adult (juvenile feeding on a food source different than that of the adult has been reported for the non-symbiotic nudibranch *Doridella obscura* by Perron and Turner, 1977), 3) by ingestion of the symbiont that is accomplished by some sort of perceptual mechanism, such as chemotaxis (Kinzie, 1974; Fitt and Trench, 1983), that acts to bring host and symbiont together.

Kempf (1982) has shown that the digestive cells of larvae of *M. pilosa* and possibly *P. ianthina* are capable of phagocytosing the unicellular alga *Pavlova (Monochrysis) lutheri.* This phagocytic potential offers a mechanism that would allow larval inception of the symbiosis in these species. The lack of algal cells in pre-metamorphic larvae of *B. major* and the presence of algae in the tissues of newly metamorphosed juveniles after feeding on adult prey (unpub. pers. obs.) suggest that each new generation of this nudibranch is re-infected with symbiotic algae obtained from its prey after metamorphosis.

Inception and evolution of nudibranch-zooxanthellae symbioses

The evolution of intracellular symbioses (Richmond and Smith, 1979; Smith, 1979; Taylor, 1979) and algal-invertebrate symbioses in particular (Trench, 1979,

1980) have been recently reviewed. Trench (1980) pointed out that examination of existing symbioses will allow insight into the evolutionary origin(s) of the many possible levels of host-symbiont integration, but that one must take care not to assume that patterns seen among extant associations mimic evolutionary pathways. In the case of nudibranch symbioses with zooxanthella(ae?), Rudman (1982) has recently speculated that these associations are of a "secondary" nature and arose through the mechanism of a selective opportunity afforded by the nudibranch hosts feeding on prey symbiotic with the alga(ae?). This hypothesis was based on his observation that nearly all the nudibranch species he examined were known to feed on such organisms. The present study has shown that adult *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* maintain an intracellular and possibly mutualistic symbiosis (however, see below) with the zooxanthella *Symbiodinium microadriaticum.* That these nudibranchs do not feed on prey containing these symbionts suggests that the evolutionary inception of their symbiotic association was not of a secondary, opportunistic nature. Thus, it appears that there are at least two pathways by which nudibranch-zooxanthella symbioses could have been established; either 1) via the opportunity afforded by the host feeding directly on prey that are symbiotic with this dinoflagellate (Rudman, 1982) or 2) by inadvertant (at least on the part of the host) ingestion of the symbiont while feeding.

In the first instance, ample opportunity for the selection of a symbiosis between the potential host and *Symbiodinium microadriaticum* would occur as the potential host feeds on organisms symbiotic with the alga. Additionally, the alga might be predisposed to a symbiotic relationship with the potential host by virtue of such an association existing between the alga and the nudibranch's prey.

If, as appears to have been the case for *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina,* the potential host's only exposure to *Symbiodinium microadriaticum* was by inadvertent ingestion, and therefore possibly rare, the mechanism(s?) by which a mutualism might have been established are not as clear. A predisposition of *Symbiodinium microadriaticum* to symbiotic associations, as mentioned above, may have been a factor; however, perhaps the suggestion of Goetsch (1924) and Goetsch and Scheuring (1927) also offers a plausible answer. They suggested that mutualistic relationships could have arisen from symbioses that were initially beneficial to the symbiont, but detrimental to the host (*i.e.,* of a parasitic nature). In such associations that did not become extinct because of mortal virulence of the symbiont, selection for host immunity, or some other cause, it seems likely that selection would tend to favor modification of the association such that the host benefits to the symbiont would be maintained or enhanced and the pathological effects of the symbiont on the host would be decreased. Under such circumstances it is not hard to envision selection leading to the establishment of a mutualism.

It has been suggested above that the nudibranch symbioses examined in this investigation might be mutualisms; however, the apparent digestion of symbionts by three species of nudibranchs leads one to ask if their symbioses should really be viewed as mutualistic. Rather than interactions involving mutual benefits, it appears that such hosts have "gained the upper hand" in the association and now hold the algal symbiont in bondage. It is, of course, possible that the clonal nature of the symbiont may offset the apparent disadvantages of digestion by the host, the alga asexually reproducing far more copies of its genome than the host digests.

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